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L18 ANSWER 8 OF 12 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1995:4656 BIOSIS
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TITLE: Ultrarapid HLA-DR4 oligotyping using a triple primer solid phase PCR method that is suitable for a routine diagnostic laboratory.
AUTHOR(S): Eibschutz, Barry; Taniguchi, Atsuo; Carson, Dennis A.; Kohsaka, Hitoshi
CORPORATE SOURCE: Univ. California, San Diego, La Jolla, CA 92093-0663 USA
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TITLE: Miniature solid-phase PCR and hybridization assay of HLA-B27 on an individual microbead.
AUTHOR(S): Soukka, T. (1); Harma, H. (1); Sjoroos, M.; Tarkkinen, P.

## 1013

RAPID AUTOMATED DETERMINATION OF HLA CLASS II DR4 SUBTYPES ASSOCIATED WITH SUSCEPTIBILITY TO RHEUMATOID ARTHRITIS. Vivian H. Gersuk, Barbara S. Nepom, Gerald T. Nepom, Virginia Mason Research Center, Seattle, WA 98101, Sharon A. Kochik, Nicolass Vermeulen, Paul C. Harris, MicroProbe Corporation, Bothell, WA 98021.

Genetic susceptibility to RA in Caucasians corresponds to HLA-DR4-associated genes which encode the "Dw4/14 shared epitope" (DRB1*0401/0404); detection of these genes may be predictive for distinguishing patients with a high likelihood of developing severe, erosive disease. Current methods for identifying individuals positive for these susceptibility genes are not optimal in terms of speed, sensitivity, and specificity. We wish to report the development of a semi-automated HLA-typing system for the rapid determination of these RA susceptibility alleles.

The assay system is a novel adaptation of conventional techniques and is composed of two steps. First, genomic DNA is amplified by the polymerase chain reaction (PCR) using DR4 group-specific primers, designed to recognize all DR4 alleles. Second, the DR4 amplification products are subtyped on an automated instrument using a solid phase, non-radioactive, allele-specific hybridization assay. Positive and negative procedural controls are utilized for quality assurance. The hybridization/detection assay is complete in less than one hour.

To test the sensitivity and specificity of this assay 214 randomly selected genomic DNA samples (including Dw4, Dw14, other DR4 subtypes and non-DR4 types), previously typed by conventional methods, were assayed. Twelve of the tested samples failed to produce a PCR product, probably due to sample degradation. Of the remaining 202 samples, all 202 were correctly identified, including 37 Dw4 samples, 24 Dw14 samples, 13 samples heterozygous for Dw4/14, and 128 samples composed of other DR4 (21) and non-DR4 (107) types. Since no samples were misidentified, the assay appears to be extremely specific. The sensitivity limit of the hybridization assay was determined to be approximately 1 ng of input PCR product. This system improves upon conventional methodology in speed, simplicity, sensitivity, and specificity, is suitable for routine clinical use, and may be conveniently modified for the rapid identification of other class II as well as non-HLA genotypes.

This work was supported, in part, by MicroProbe Corporation, a for-profit biotechnology company.

## 1014

ULTRARAPID HLA-DR4 OLIGOTYPING USING A TRIPLE PRIMER SOLID PHASE PCR METHOD THAT IS SUITABLE FOR A ROUTINE DIAGNOSTIC LABORATORY. Barry Eibschutz, Atsuo Taniguchi, Dennis A. Carson, Hitoshi Kohsaka, University of California, San Diego, La Jolla, California 92093-0663.

Seropositive RA is associated with the HLA-DR subtypes DRB1*0101, *0401, *0404, and *0408, but not *0402 or *0403. Whether an individual inherits one or two of these alleles correlates with RA disease severity and outcome. It has therefore been suggested that HLA-DR subtyping be part of the work-up of newly diagnosed RA patients. Unfortunately, current methods of HLA-DR subtyping are unsuitable for the routine diagnostic laboratory, because they require electrophoresis, the use of hazardous reagents, and are difficult to interpret reliably. To overcome this problem, we have developed a novel typing method termed triple primer solid phase PCR. The technique uses a 5'-fluoresceinated primer and a 3'-unlabelled primer, but is performed in microtiter ELISA plates, previously coated with a third, covalently attached oligonucleotide complementary to the fluoresceinated strand of the expected PCR product. During the course of the in situ PCR reaction, the fluoresceinated PCR product attaches to the microtiter plates. After simple washing away of unreacted primer, bound product is detected in a spectrophotometer. There is no requirement for transfer of the product, electrophoresis, or radioactivity. The use of three primers assures sensitivity and specificity, permitting the detection of a single base pair change in total human genomic DNA. Using this method, we can distinguish HLA-DRB1*0401 and *0404 from other HLA types, simply and rapidly (about 2 hrs). Based on the increasing evidence that certain DR4 alleles correlate with RA disease severity, this ultrarapid method of DR4 oligotyping could be utilized routinely in clinical assessment.

not actually amplifying on solid phase. (primer on solid) – accidental 102?

## 1015

HLA DRB1 DISEASE-RELATED SUBTYPES IN RHEUMATOID ARTHRITIS (RA). ASSOCIATION WITH SUSCEPTIBILITY BUT NOT SEVERITY. ME Suarez-Almazor, T Shao, R Nelson, E Pazderka, AS Russell, W Maksymowych, University of Alberta, Edmonton, AB, CANADA, T6G 2R7.

The association of HLA DR4 with RA has been well established. Doubts remain however as to whether DR4 is a marker for susceptibility, severity or both. The most recent evidence has pointed to specific HLA DRB1 subtypes as responsible for the association. The purpose of this study was to determine the relationship of DR1, DR4 and DR4 subtypes with severity of the disease in a community-based sample of patients with RA, given that most prior studies have been conducted in highly selected populations with severe disease. The study was based on a cohort of 103 Caucasian patients with an onset of disease in 1985, and 6 to 7 years of disease at the time of the study. HLA status was determined using sequence-specific PCR; 58 patients (57%) were DR4 positive, compared to the expected frequency in North American Caucasians (25-30%, p=0.002); 65 patients (63%) carried either a DR4 or DR1 allele. No significant differences were observed in disease activity, joint counts, radiological scores or functional status when comparing DR4 positive and negative patients, or DR1 and/or DR4 positive with DR1 and DR4 negative individuals. Only 9% of the DR4+ patients did not carry a disease-related DR4 subtype. After excluding these patients from the analysis, still no significant differences were observed in the severity of disease among patients carrying 1, 2 or no disease-related alleles:

| | No alleles (45) | 1 allele (39) | 2 alleles (18) | p value |
|---|---|---|---|---|
| N° swollen joints | 4.8 (5.9) | 4.5 (5.9) | 4.2 (4.5) | 0.91 |
| N° tender joints | 3.4 (3.9) | 3.5 (4.1) | 3.4 (3.5) | 1.0 |
| Radiological score | 6.0 (6.2) | 8.6 (11.6) | 4.9 (4.6) | 0.22 |
| Functional status index (MHAQ) | .44 (.43) | .57 (.48) | .45 (.45) | 0.36 |

In this setting HLA DR1 and HLA-DR4 disease-related subtypes do not appear to have any predictive value to determine subsequent severity. In this cohort, rheumatoid factors were better predictors of severity. The results of our study suggest that, in community-based settings, which include patients with milder disease, DR1/DR4 disease-linked alleles increase susceptibility for RA, but cannot be used as markers for disease severity.

## 1016

PROGNOSIS IN RHEUMATOID VASCULITIS WITH NEUROPATHY. X. Puéchal, G. Saïd*, P. Hilliquin, J. Costé, S. Perrot, C. Job-Deslandre, C.J. Menkès. Hôpital Cochin, 75674 Paris Cedex 14; *Hôpital de Bicêtre, 94270 Le Kremlin-Bicêtre, France.

***Objective.*** To examine survival and to identify factors at diagnosis that predicted subsequent survival among patients with rheumatoid arthritis (RA) and vasculitic neuropathy.

***Methods.*** 32 patients with RA and peripheral neuropathy were studied, in whom necrotizing vasculitis was found in nerve and/or muscle biopsy specimens. Patients were followed for 14 months to 22 years (median, 7.2 years). Survival was evaluated and the prognostic value of clinical, biological and pathological features was assessed by Cox's proportional hazards model. A prognostic index based on the significant variables was devised to estimate the probability of survival for any individual patient.

***Results.*** During the study period, 14 patients (44 %) died. The descending order of significance of factors related to mortality was a clinical cutaneous vasculitis (p=0.0003), the diffusion of the neuropathy to 3 or 4 limbs (p=0.03) and a depressed level of C4 (p<0.05). Advancing age tended also to predict decreased survival (p=0.11). Motor involvement of the neuropathy did not appear to be an independent prognostic factor. Mononeuritis multiplex was not associated with poorer survival than distal symmetrical sensorimotor neuropathy. Our prognostic index indicated a wide range for the 5-year probability of survival from less than 1 % to 93 %.

***Conclusion.*** Clinically associated cutaneous vasculitis, wide diffusion of the neuropathy and a depressed level of C4 were three independent variables which best predicted mortality. There was a wide range for the probability of survival according to the pattern of these prognostic factors. Our prognostic index needs to be confirmed. However, it may even in this preliminary stage help physicians to adapt therapy according to the prognosis. It could also be used in further clinical trials to stratify patients for more or less aggressive therapeutic regimens.

## 1017

HLA-B27 AS A MARKER FOR DEVELOPING SUBLUXATIONS OF THE CERVICAL SPINE IN RA. W Ollier, L Pepper, W Thomson, ARC, ERU, University of Manchester, UK, A Young, St Albans Hospital, Hertfordshire, UK.

The development of subluxations of the cervical spine is a potentially serious complication of rheumatoid arthritis (RA) which in some cases can be life threatening. Erosive changes can be in either or both atlanto-axial and sub-axial joints. RA is strongly associated with HLA-DR4 and with a consensus sequence within the third hypervariable region of DRB1. Other MHC molecules, either independently or in haplotypic association with DR4 may influence the outcome of disease expression in RA.

An earlier retrospective study using serological typing (Jaraquemada et al., Ann Rheum Dis 1986;45:627-636) suggested that HLA-B27 may be at raised frequency in RA patients with subluxations of the cervical spine. We have now examined a cohort of 78 RA patients satisfying ARA criteria and who have been longitudinally studied from onset. These patients have been monitored for three years and radiologically assessed for subluxations of the cervical spine. DNA samples were typed for HLA-DR4 and all HLA-B27 variants using PCR-oligonucleotide probing. Control HLA data was available on 135 healthy subjects.

In all patients a DR4 frequency of 71.8% and a B27 frequency of 25.5% was observed compared to control frequencies of 36.6% and 10% respectively. An increase in HLA-B27 frequency was observed in RA patients with subluxations (40%) compared to those without (18.8%). HLA-B27 frequency particularly high in patients with sub-axial changes (50%). No particular HLA-B27 or DR4 variant was associated with subluxation. HLA-B27 positive RA patients have approximately twice the risk of developing subluxation of the cervical spine compared with B27 negative patients (RR 1.9, 95% CI 1.0 - 3.6). The risk is even higher for the development of sub-axial subluxation (RR 2.9, 95% CI 1.3 - 6.3). As yet no biological explanation can be given for these associations. HLA B27 typing of RA patients may be a useful prognostic indicator for the development of subluxations of the cervical spine in RA.

## 1018

HLA-DRB1 ANALYSIS IN ITALIAN RHEUMATOID ARTHRITIS PATIENTS : CORRELATION WITH THE EROSIVE DISEASE. Pierluigi Macchioni, Carlo Salvarani, *Wilma Mantovani, Fulvia Rossi, Italo Portioli. Unità Reumatologica, USL9, Reggio Emilia; *Laboratorio Chimico Clinico,USL28,Bologna,42100,ITALY.

We evaluated the contribution of the HLA-DRB1*04 variants in the clinical and radiological evolution of rheumatoid arthritis in a group of 24 italian patients.

DNA-based HLA typing for DRB1, DQB1 and DPB1 was performed in 24 DR4 positive Italian RA patients and 30 DR4 healthy controls. All the patients had RA according to the ACR 1987 criteria and were followed by our department for a minimum period of 4 years. The records of the patients were reviewed for the presence of RF, ANA positivity, extra-articular features, duration of the disease, previous therapy and adverse effects from the treatment. Radiographs of hands and feet were available for all the patients. They were evaluated by three different observers for the presence of erosions and graduated according to the Larsen score. No differences in the distribution of the HLA-DRB1*04 variants was found comparing diseased and healthy subjects. RA patients carrying the HLA-DRB1*0401 gene had a significant increase in the Larsen (70.5 vs 48.6, p=0.01) and in the erosive score (12.5 vs 6.5, p=0.016) when compared with patients without this gene. The relative risk of having more then 10 joints eroded were 2.17 for the patients carryng the HLA-DRB1*0401 gene. No differences were present in the sex distribution, disease duration, RF positivity or extraarticular disease comparing the two groups of patients. In Italian patients the HLA-DRB1*0401 gene seems to be a marker more erosive form of RA.

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(1); Lovgren, T. (1)

CORPORATE SOURCE: (1) Department of Biotechnology, University of Turku, Turku

Finland

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Juliet Einsmann
AU 1634
703 306 5824
Office: CM1-12D15
Mailbox: CM1-12E12

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102(a)

## ARRAYED PRIMER EXTENSION AS A TOOL FOR DNA DIAGNOSTICS

*Metspalu A.*
Institute of Molecular and Cell Biology, University of Tartu; Children's Hospital; Estonian Biocentre and Asper Ltd., TARTU, ESTONIA

Human Genome Project (HGP) is expected to produce among others the DNA sequence of a human genome. The long-term effect of the HGP is envisioned to be an understanding of gene interactions that occurs during both developmental and disease processes. Major paradigm change is needed if we want to use the vast amount of information being contained in human genome. We need a new technology suitable to analyze genomes instead of genes. One possibility is to use of DNA chips. There are several DNA chip concepts in development today, including DNA arrays for monitoring of gene expression and oligonucleotide arrays for mutation analysis on DNA resequencing. I will present a DNA-chip concept called APEX – Arrayed Primer EXtension. According to this method array of oligonucleotides is immobilized to silanized microscope cover glass via 5' amino link. Oligonucleotides (15-25 mers) are selected from known sequence of the gene(s) so that the nucleotide to be analyzed in patient DNA is next from the 3' end of the immobilized oligonucleotide. By annealing of the patient DNA to the oligonucleotides on the chip and performing a single nucleotide primer extension step with DNA polymerase and fluorescently labelled ddNTP-s (different dye for the each) we can elongate the immobilized primer by one nucleotide complementary to the patient DNA. As a template we are using double strand PCR product amplified from the patient material and fragmented by uracil-N-glycosylase before annealing. Custom-built CCD based imager is used for four color detection based on total internal reflection fluorescence. Time required for APEX is 5-10 min and for imaging one minute. We applied APEX for the identification of 10 most common point mutation in the human β-globin gene causing β-thalassemia in Mediterranean region. Results are demonstrating that all mutations either homozygous or heterozygous state can be detected. To demonstrate the full power of the APEX technology a fragment of p53 gene (DNA binding region) was resequenced from both strands simultaneously. Compared to "hybridization only" approach to "hybridization plus enzyme" method the later has much higher signal to noise ratio making possible to analyze heterozygous genomes and somatic mutations.
In conclusion we have developed the integrated system consisting of oligonucleotide array production, template preparation, reaction scheme and versatile four-color fluorescent imager to perform SNP analysis, mutation detection and DNA resequencing.

## MINIATURE SOLID-PHASE PCR AND HYBRIDIZATION ASSAY OF HLA-B27 ON AN INDIVIDUAL MICROBEAD

*Soukka T.[1], Härmä H.[1], Sjöroos M.[2], Tarkkinen P.[1], Lövgren T.[1]*
1) Department of Biotechnology, University of Turku (Finland)
2) Department of Virology, University of Turku (Finland)

Miniaturized solid-phase nucleic acid amplification on microbeads has been studied to combine separate amplification and hybridization procedures. The obvious advantage of this method is elimination of amplification liquid handling and product collection step enabling shorter assay time. Further, miniaturized solid-phase assay favors automatization and improves cost effectiveness.
Solid-phase primers were covalently attached at 5' end onto the carboxyl acid surface of monosized, porous microparticles, 50 ?m in diameter. Allele-specific polymerase chain reaction was carried out to amplify a 144 bp region of HLA-B27 alleles, associated with ankylosing spondylitis, onto an individual microparticle. The double-stranded amplified product was denatured and detected by hybridization assay employing product specific oligonucleotide probe labeled with luminescent terbium chelate. Time-resolved fluorescence was measured on the surface of a single bead.
Amplification in 500 nL volume from 0.2–0.4 ng of human genomic DNA produced more than 2 x $10^8$ copies per particle. Since the detection limit was less than 1 x $10^6$ molecules over 200-fold signal to background ratio was achieved. The developed method was succesfully employed to analyze clinical samples. The combined solid-phase method has shown to allow rapid and sensitive, miniaturized assay format, which can be further multiplexed utilizing categorized beads.

not labeled primers.

## GENE EXPRESSION MEASUREMENT USING DNA ARRAYS: SENSITIVITY ISSUES AND ADVANTAGES OF NYLON MICROARRAYS FOR SMALL SAMPLES.

*Bertucci F.[1], Loriod B.[2], Granjeaud S.[3], Houlgatte R.[3], Peck K.[4], Jordan B.R.[3], Nguyen C.[3]*
1) Tagc, Institut Paoli-Calmettes, Marseille (France)
2) Tagc, Ciml, Marseille (France)
3) Tagc, Ciml, Marseille (France)
4) Institute of Biomedical Sciences, Academia Sinica, Taipei (Taiwan)

Large-scale measurement of gene expression using hybridisation of complex probes to arrays of cDNA inserts or oligonucleotides is an increasingly used technique both in the academic and in the corporate sector. The method can provide quantitative measurement of expression levels of thousands of genes in different tissues or in normal versus pathological samples, with good reproducibility and few artefacts in carefully controlled experiments. Arrays are widely used for large-scale expression profiling, using various implementations: macroarrays in which DNA is spotted onto Nylon membranes of relatively large dimensions (with radioactive detection); microarrays on glass slides and oligonucleotide chips, both used with fluorescent probes, or Nylon microarrays with colorimetric detection as recently described. The sensitivity of these systems is an important issue in a number of situations where biological material is very limited. The small physical dimensions of miniaturised systems allow small hybridisation volumes (2 to 100 µl) and provide high probe concentration, in contrast to macroarrays. We show however that actual sensitivity is in fact similar for all these systems, and that this is mostly due to the very different amounts of target material present on the respective arrays. We then demonstrate that the combination of Nylon microarrays with 33P-labelled radioactive probes provides extremely good sensitivity, making it possible to perform expression profiling experiments using -microgram amounts of unamplified total RNA. These may be obtained from very small biological samples. This has important implications in basic and clinical research.